

RESEARCH ARTICLE

Ralstonia solanacearum Requires PopS, an Ancient AvrE-Family Effector, for Virulence and To Overcome Salicylic Acid-Mediated Defenses during Tomato Pathogenesis

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ABSTRACT During bacterial wilt of tomato, the plant pathogen *Ralstonia solanacearum* upregulates expression of *popS*, which encodes a type III-secreted effector in the AvrE family. PopS is a core effector present in all sequenced strains in the *R. solanacearum* species complex. The phylogeny of *popS* mirrors that of the species complex as a whole, suggesting that this is an ancient, vertically inherited effector needed for association with plants. A *popS* mutant of *R. solanacearum* UW551 had reduced virulence on agriculturally important *Solanum* spp., including potato and tomato plants. However, the *popS* mutant had wild-type virulence on a weed host, *Solanum dulcamara*, suggesting that some species can avoid the effects of PopS. The *popS* mutant was also significantly delayed in colonization of tomato stems compared to the wild type. Some AvrE-type effectors from gammaproteobacteria suppress salicylic acid (SA)-mediated plant defenses, suggesting that PopS, a betaproteobacterial ortholog, has a similar function. Indeed, the *popS* mutant induced significantly higher expression of tomato SA-triggered pathogenesis-related (PR) genes than the wild type. Further, pretreatment of roots with SA exacerbated the *popS* mutant virulence defect. Finally, the *popS* mutant had no colonization defect on SA-deficient NahG transgenic tomato plants. Together, these results indicate that this conserved effector suppresses SA-mediated defenses in tomato roots and stems, which are *R. solanacearum*'s natural infection sites. Interestingly, PopS did not trigger necrosis when heterologously expressed in *Nicotiana* leaf tissue, unlike the AvrE homolog DspE_{pec} from the necrotroph *Pectobacterium carotovorum* subsp. *carotovorum*. This is consistent with the differing pathogenesis modes of necrosis-causing gammaproteobacteria and biotrophic *R. solanacearum*.

IMPORTANCE The type III-secreted AvrE effector family is widely distributed in high-impact plant-pathogenic bacteria and is known to suppress plant defenses for virulence. We characterized the biology of PopS, the only AvrE homolog made by the bacterial wilt pathogen *Ralstonia solanacearum*. To our knowledge, this is the first study of *R. solanacearum* effector function in roots and stems, the natural infection sites of this pathogen. Unlike the functionally redundant *R. solanacearum* effectors studied to date, PopS is required for full virulence and wild-type colonization of two natural crop hosts. *R. solanacearum* is a biotrophic pathogen that causes a nonnecrotic wilt. Consistent with this, PopS suppressed plant defenses but did not elicit cell death, unlike AvrE homologs from necrosis-causing plant pathogens. We propose that AvrE family effectors have functionally diverged to adapt to the necrotic or nonnecrotic lifestyle of their respective pathogens.

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Plant-pathogenic bacteria cause destructive diseases that limit crop production worldwide. Many Gram-negative phytopathogenic bacteria use a type III secretion system (T3SS) to inject effector proteins into host cells. These generally modulate host immunity and physiology for pathogenesis (1–4). Individual effectors rarely contribute measurably to virulence but rather function as a consortium (5). Because of their redundancy and subtle biological activities, the functions of individual type III (T3) effectors remain largely unknown.

Plant immune systems have evolved complex signaling responses to defend against microbial invasion. Plants use specific protein receptors to detect conserved features of pathogen products such as flagellin, chitin, lipopolysaccharide, and elongation

factor Tu (EF-Tu); perception of these microbe-associated molecular patterns (MAMPs) triggers basal immunity (1). In addition, plant *R* genes specifically recognize pathogen effectors. The plant hormone salicylic acid (SA) is a major defense signal molecule (6), and upon recognition of a pathogen, SA production induces basal immune responses such as callose deposition (7, 8). Accumulation of SA in plants also induces expression of pathogenesis-related (PR) defense genes to resist microbial infection and sometimes triggers rapid host cell death (9, 10). Studies of a few genera in the gammaproteobacteria have revealed how pathogen T3 effectors disrupt immune signaling and suppress SA-mediated defenses (5, 11, 12).

The AvrE family of effectors is well conserved across agricul-

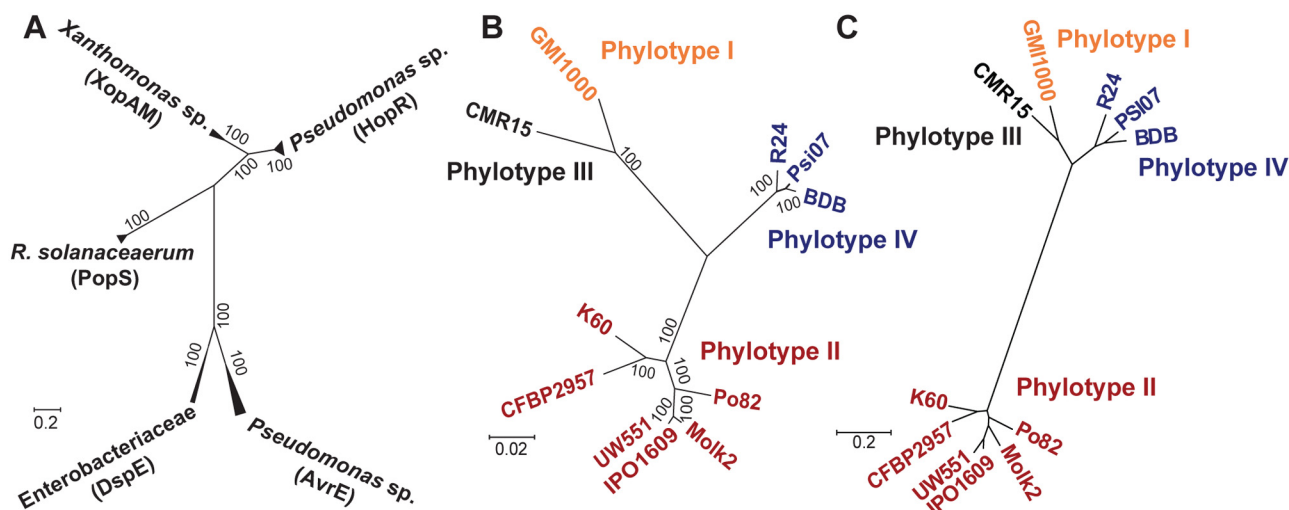


FIG 1 PopS, an ancient core T3 effector in plant-pathogenic *Ralstonia* species, forms a distinct clade of the AvrE/DspE/HopR effector family. Phylogenetic trees were based on comparative analysis of whole genomes of 11 representative sequenced strains. (A) PopS, HopR (XopAM) from *Xanthomonas* spp. and *Pseudomonas syringae* pathovars, AvrE from *P. syringae* pathovars, and DspE/WtsE from enterobacteria; (B) PopS from sequenced plant-pathogenic *Ralstonia*; (C) *R. solanacearum* species complex. For panels A and B, amino acid sequences were aligned using CLUSTAL-W, from which a maximum-likelihood phylogenetic tree was created with MEGA5. The percentage of replicate trees in which the individual orthologs clustered together in the bootstrap test (200 replicates) is noted at each branch. For panel C, a phylogenetic tree of the *R. solanacearum* species complex was derived from a MUM index (MUMi) distance matrix of whole-genome sequences using the neighbor-joining clustering method.

turally important phytobacteria, including enterobacteria, xanthomonads, and pseudomonads (gammaproteobacteria) and *Ralstonia* spp. (betaproteobacteria) (13). Effectors in this family, which includes AvrE, DspE, and WtsE, induce host cell death and suppress defense signaling (12–14). AvrE from *Pseudomonas syringae* pv. *tomato* DC3000 and its ortholog DspE_{Ed} from *Erwinia amylovora* promote pathogen growth and overcome plant immunity by inhibiting SA-mediated defense responses (12). Despite their broad relevance to the interactions of plant-pathogenic bacteria with their hosts (13), little is known about AvrE-like effectors outside plant-pathogenic gammaproteobacteria.

The bacterial wilt pathogen *R. solanacearum* is responsible for diseases of many crops in tropical and subtropical climates worldwide. This bacterium enters plant roots from the soil and colonizes the host vasculature, which eventually leads to wilt and plant death (15). *R. solanacearum* requires a T3SS for root and stem invasion and colonization (16), and T3SS-deficient strains are essentially unable to wilt host plants (16). The *R. solanacearum* genome encodes an extensive effector repertoire (2, 17). Mutants lacking individual effectors generally do not have virulence defects (18), likely because the effectors have redundant functions (3, 5). The defense-suppressing functions of the individual effectors during the infection cycle remain unknown.

We previously used gene expression analysis to define the *R. solanacearum* *in planta* transcriptome, the set of bacterial genes expressed during growth in wilting tomato plant stems (19). An orthologous gene encoding an AvrE-family effector was expressed *in planta* in two ecologically and phylogenetically distinct strains. This locus (RRSL_03375 in strain UW551 and RSp1281 in strain GMI1000) encodes a secreted T3 effector in the AvrE/DspE/HopR protein family (20), herein named PopS. Relative to expression in rich culture medium, UW551 and GMI1000 upregulate *popS* *in planta* 14- and 8-fold, respectively (19). Expression of *popS* is de-

pendent on HrpB, the transcriptional activator of the T3SS and its effectors (21–23). Most effector genes are upregulated *in planta* via HrpB (19, 21, 24).

This study characterizes the role of PopS throughout the tomato infection process. We determined that this effector, which has ancient roots in the *R. solanacearum* species complex, is required for normal host colonization and virulence on multiple *Solanum* spp. crop hosts. PopS was dispensable for virulence on a weed, *Solanum dulcamara*, suggesting that it has species-specific virulence activity within the genus *Solanum*. PopS forms a unique clade in the AvrE family of effectors. Although it is highly divergent from its closest orthologs, we found that PopS retains the function of suppressing SA-mediated plant defenses. In contrast, PopS did not cause plant cell death or necrosis as do the AvrE-family proteins of necrosis-causing pathogens, such as DspE_{pc} and WtsE from *Pectobacterium carotovorum* subsp. *carotovorum* and *Pantoea stewartii* subsp. *stewartii*. Together, our phylogenetic, virulence, and gene expression data suggest that PopS suppresses SA-mediated host defenses but lacks the ability to cause cell death, which may have helped this pathogen adapt to its nonnecrotic lifestyle.

RESULTS

PopS is a conserved, vertically inherited T3 effector in the *R. solanacearum* species complex. The AvrE effector family is widely present among plant-pathogenic bacteria (13). PopS most closely resembles HopR in *Pseudomonas* spp. and *Xanthomonas* spp. (also known as XopAM; <http://www.xanthomonas.org/t3e.html>) (13), which were, respectively, 25 to 26% and 27 to 28% identical to PopS at the amino acid level. To understand the phylogenetic relationships among these proteins, we developed a maximum-likelihood phylogenetic tree in MEGA5 based on protein sequences of available PopS, AvrE, DspE, and HopR effector or-

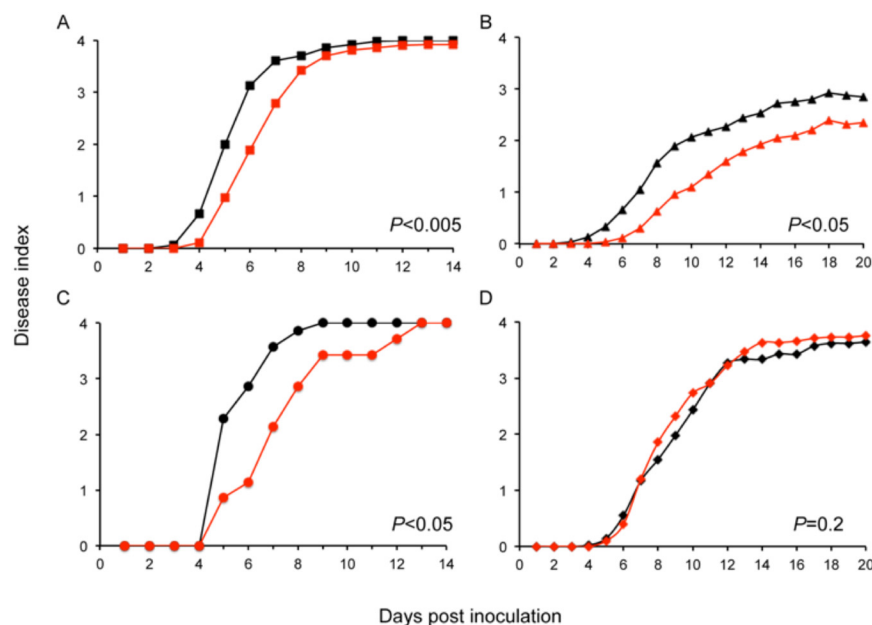


FIG 2 Virulence of UW551 *popS* mutant on diverse plant hosts. *R. solanacearum* wild-type strain UW551 (black) and the *popS* mutant (red) were inoculated by pouring a bacterial suspension onto unwounded roots of wilt-susceptible tomato (A) and wilt-resistant tomato (B) (Bonny Best and H7996, respectively), potato (cv. Russet Norkotah) (C), and *Solanum dulcamara* (bittersweet nightshade) (D). Symptoms were rated daily using a disease index scale of 0 to 4 (0, healthy; 1, 1 to 25% of leaves wilted; 2, 26 to 50% of leaves wilted; 3, 51 to 75% of leaves wilted; 4, 76 to 100% of leaves wilted). Each point represents the mean disease index from three independent experiments, each containing 16 plants per treatment (A, B, and D), or one experiment (C). The *popS* mutant was significantly less virulent than the wild type on susceptible ($P < 0.005$; repeated-measures ANOVA) and resistant tomato and potato ($P < 0.05$) but not on bittersweet nightshade ($P = 0.2$).

thologs (Fig. 1; also, see Table S1 in the supplemental material) (25). The resulting robust tree, based on 58 amino acid sequences, demonstrated that PopS forms a unique clade in the AvrE/DspE/HopR effector family and that this large family of effectors forms three distinct clusters: AvrE/DspE, HopR, and PopS (Fig. 1A).

The *R. solanacearum* species complex is divided into four subgroups called phylotypes (26). A single copy of *popS* is found in the genomes of all 12 sequenced strains in the *R. solanacearum* species complex, which includes the blood disease bacterium (BDB) and the clove pathogen *Ralstonia syzygii*, both fastidious pathogens with limited host ranges (17, 27–33). A maximum-likelihood phylogenetic tree of genes encoding the *R. solanacearum* AvrE-like proteins indicated that *popS* sequences share the phylogeny of the species complex as a whole (Fig. 1B).

To test the hypothesis that *popS* has been vertically inherited over the evolution of the *R. solanacearum* species complex, we developed a neighbor-joining tree based on a MUMi distance matrix for whole-genome phylogenetic analysis (Fig. 1C) (17, 34). The MUMi distance matrix and phylogenetic tree of the sequenced plant-pathogenic *Ralstonia* strains mirror the previously determined phylogeny of the species complex (17). Moreover, the phylogenetic tree of PopS itself mirrors the whole-genome MUMi tree of the species complex (Fig. 1B and C) (26). There is no indication of interstrain movement of *popS* via horizontal transfer, suggesting that PopS is an ancient core effector in the species complex. This conserved, vertically inherited gene is potentially useful for typing strains and identifying their phylogenetic positions in the *R. solanacearum* species complex.

***R. solanacearum* T3 effector PopS is required for full virulence on several hosts.** To study the virulence function of this effector, we disrupted *popS* in strain UW551 (phylotype II, sequevar 1) via allelic exchange to create strain UW551 *popS::Km^r* (referred to here as the *popS* mutant). This mutant grew indistinguishably from the wild type (WT) in culture medium (data not shown), indicating that *popS* is not required for *in vitro* growth. The *popS* mutant retained wild-type ability to grow on sucrose as the sole carbon source, indicating that insertion of the *Km^r* cassette did not disrupt expression of the *scrK* sucrose kinase gene (RRSL_03374) immediately downstream of *popS*. Quantitative reverse transcriptase PCR (qRT-PCR) analysis revealed that UW551 WT, but not the *popS* mutant, accumulates *popS* transcript when grown in minimal medium, confirming that *popS* is not expressed in the mutant strain (data not shown). To test the hypothesis that PopS contributes to bacterial wilt virulence, we used a naturalistic soil soak virulence assay to compare wilt disease progress of UW551 WT and the *popS* mutant on susceptible and moderately resistant tomato (*Solanum lycopersicum* cv. Bonny Best and H7996, respectively), susceptible potato (*S. tuberosum*

cv. Russet Norkotah), and a natural weed host, *S. dulcamara* (bittersweet nightshade) (19). Briefly, pots containing unwounded plants were soaked with bacterial suspensions, and disease progress was rated daily. The *popS* mutant was delayed in virulence on potato ($P < 0.05$; repeated-measures analysis of variance [ANOVA]) and on both susceptible and resistant tomato ($P < 0.05$ and $P < 0.005$, respectively; repeated-measures ANOVA) (Fig. 2A, B, and C). The *popS* mutant had a larger virulence defect on the moderately resistant H7996, suggesting that PopS plays a larger role in virulence on resistant plants (Fig. 2A and B).

In contrast, loss of *popS* did not affect *R. solanacearum* virulence on *S. dulcamara*, an epidemiologically important weed host (35) ($P = 0.2$; repeated-measures ANOVA) (Fig. 2D). Lowering the assay temperature from a tropical 28°C to 24°C, which might favor this temperate host plant, did not change this result (data not shown), further evidence that *popS* is dispensable for wilt on *S. dulcamara*. Thus, this effector has species-specific activity, since it was necessary for wild-type disease progress on crop hosts but not on a related weed host.

PopS contributes to colonization of susceptible and resistant tomato stems. To dissect the mechanisms by which PopS contributes to *R. solanacearum* virulence, we compared the rates at which strain UW551 WT and the *popS* mutant wilted and colonized the stems of wilt-resistant tomato plants. Virulence and colonization rates for a completely T3SS-deficient *hrcC* mutant were also measured. To distinguish the stem colonization process from root invasion, tomato stems were directly inoculated through a cut

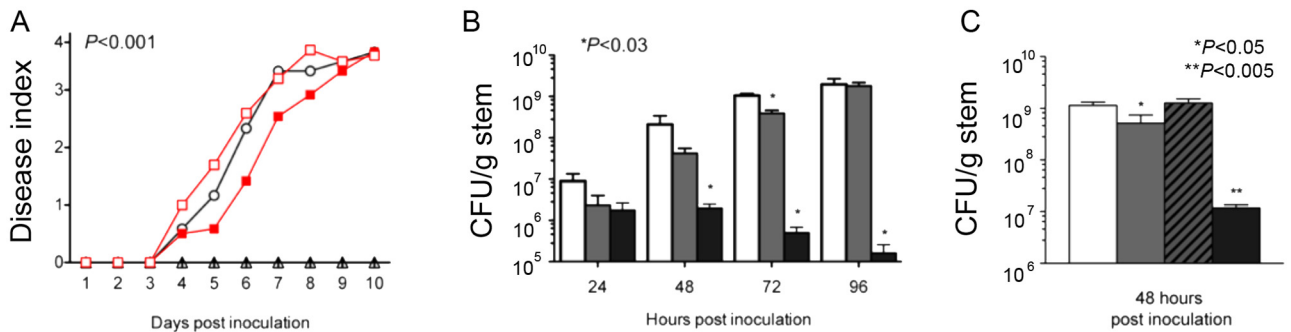


FIG 3 *R. solanacearum* requires the T3SS and effector PopS for virulence and colonization of tomato stems. (A) Stems of the resistant tomato strain H7996 were inoculated through cut petioles with 40,000 cells of WT UW551 (open black circles), a UW551 *popS* mutant (solid red squares), a UW551 *popS* mutant plus miniTn7::*popS* (UW551 *popS*-complemented strain; open red squares), and a UW551 *hrcC* mutant (open black triangles). Symptoms were rated daily using a disease index scale of 0 to 4 (0, healthy; 1, 1 to 25% of leaves wilted; 2, 26 to 50% of leaves wilted; 3, 51 to 75% of leaves wilted; 4, 76 to 100% of leaves wilted). Each point represents the average disease index ($n = 10$). The *popS* and *hrcC* mutants were significantly different from the WT and *popS*-complemented strains ($P < 0.001$; repeated-measures ANOVA). (B) H7996 stems were inoculated through cut petioles with 40,000 cells of WT UW551 (white), a UW551 *popS* mutant (gray), or a UW551 *hrcC* mutant (black). Values are average bacterial population sizes (CFU/g stem) of four plants, which were determined at 24, 48, 72, and 96 hpi. Error bars indicate standard errors; asterisks represent statistically significant differences between wild-type UW551 and the *popS* or *hrcC* mutant (Mann-Whitney test). (C) Plant stems from susceptible tomato (cv. Bonny Best) were inoculated through cut petioles with 40,000 cells of WT UW551 (white), the *popS* mutant (gray), the *popS*-complemented strain (gray with black lines), or the *hrcC* mutant (black). Columns represent the average bacterial population sizes (CFU/g stem) of four to five individual plants, which were determined 48 h postinoculation. Error bars indicate standard errors; asterisks represent statistically significant differences (Mann-Whitney test) between the WT and the *popS* or *hrcC* mutant or the *popS*-complemented strain.

petiole with WT UW551, the *popS* mutant, or the *hrcC* mutant, and bacterial colonization was quantified over time.

After direct petiole inoculation, the *popS* mutant was slightly delayed in virulence compared to WT UW551 (Fig. 3A) ($P < 0.001$; repeated-measures ANOVA). The *popS* mutant also colonized resistant H7996 tomato stems significantly more slowly than its wild-type parent ($P < 0.03$; Mann-Whitney test), although its population size reached wild-type levels by 96 h postinoculation (Fig. 3). Complementing the *popS* mutant by adding a single copy of *popS* under the control of its native promoter restored the ability of the *popS* mutant to both wilt and colonize tomato stems ($P < 0.05$; Mann-Whitney test) (Fig. 3). These results suggest that PopS is required for bacterial success *in planta* after the early stages of root infection. This result is congruent with a previous observation that a *popS* mutant of phylotype I strain GMI1000 had reduced fitness in eggplant leaves (36), although there are significant biological differences between the apoplast and xylem tissue.

As expected (16), the T3SS-deficient *hrcC* mutant was avirulent and did not effectively colonize either susceptible or resistant tomato stems (Fig. 3). The *hrcC* mutant never reached populations greater than 1.5×10^7 CFU/g stem on either host. Population sizes of the *hrcC* mutant declined gradually over the 4 days of the assay to 1.6×10^5 CFU/g stem.

The *popS* mutant induced higher SA defenses in plant roots. Following infection by pathogens, plant tissues accumulate SA, which induces expression of several PR defense genes (6, 9). Specifically, tomato plants upregulate the SA-mediated PR genes *PR-1a* and *PR-1b* in response to infection by *R. solanacearum* (37). Effectors AvrE in *P. syringae* pv. *tomato* DC3000 and DspE_{Ea} in *E. amylovora* both suppress plant defenses mediated by SA (12). Because UW551 PopS shares 23% amino acid identity with DspE_{Ea} and AvrE, we tested the hypothesis that it similarly suppresses SA-mediated host defense gene expression.

We measured expression of *PR-1a* and *PR-1b* in roots of moderately resistant H7996 tomato inoculated with GMI1000,

UW551, the UW551 *popS* mutant, or a water control. Twenty-four hours postinoculation, plants inoculated with wild-type strains GMI1000 or UW551, respectively, increased expression of *PR-1a* by 2.4- and 4.9-fold and *PR-1b* by 2.5- and 3.2-fold (Fig. 4A and B). This is consistent with our previous finding that UW551 triggers a faster response in H7996 than GMI1000 (37). Plants inoculated with the UW551 *popS* mutant had much higher levels of *PR-1a* (15.8-fold increase) and *PR-1b* (13.3-fold increase) than those inoculated with WT (Fig. 4A and B). This result indicates that PopS functions to suppress expression of host plant SA-mediated defense genes. Complementation of the *popS* mutant with the wild-type *popS* locus restored the ability of the mutant to suppress tomato SA-mediated defenses (see Fig. S1 in the supplemental material).

SA-treated tomato plants have increased resistance to a *popS* mutant. Because roots upregulated *PR-1a* and *PR-1b* in response to the UW551 *popS* mutant and SA induces tomato PR defense gene expression (6, 38, 39), we predicted that pretreating tomato plants with SA would specifically increase their resistance to the UW551 *popS* mutant. We primed the SA defenses by soaking the soil of unwounded susceptible tomato plants (cv. Bonny Best) with 7.5 mM sodium salicylate (for an estimated soil concentration of 0.75 mM SA/g soil) 6 h before inoculating the plants with either UW551 WT or the *popS* mutant. As predicted, plants pretreated with 7.5 mM sodium salicylate upregulated the SA-triggered defense genes *PR-1a* and *PR-1b* relative to water-treated control plants (Fig. 4C). SA treatment did not trigger expression of ethylene- or jasmonic acid-dependent defense genes *ACO5* or *pin2*, respectively (Fig. 4C), suggesting that *PR-1a* and *PR-1b* induction is specific to SA.

SA treatment delayed wilt symptom development in plants inoculated with UW551 WT by 2 days compared to water-treated controls (Fig. 4D and E). By the end of the assay, SA-treated tomato plants inoculated with WT UW551 wilted all tomato plants (Fig. 4E). This demonstrates that SA triggers defenses that increase plant resistance to *R. solanacearum*.

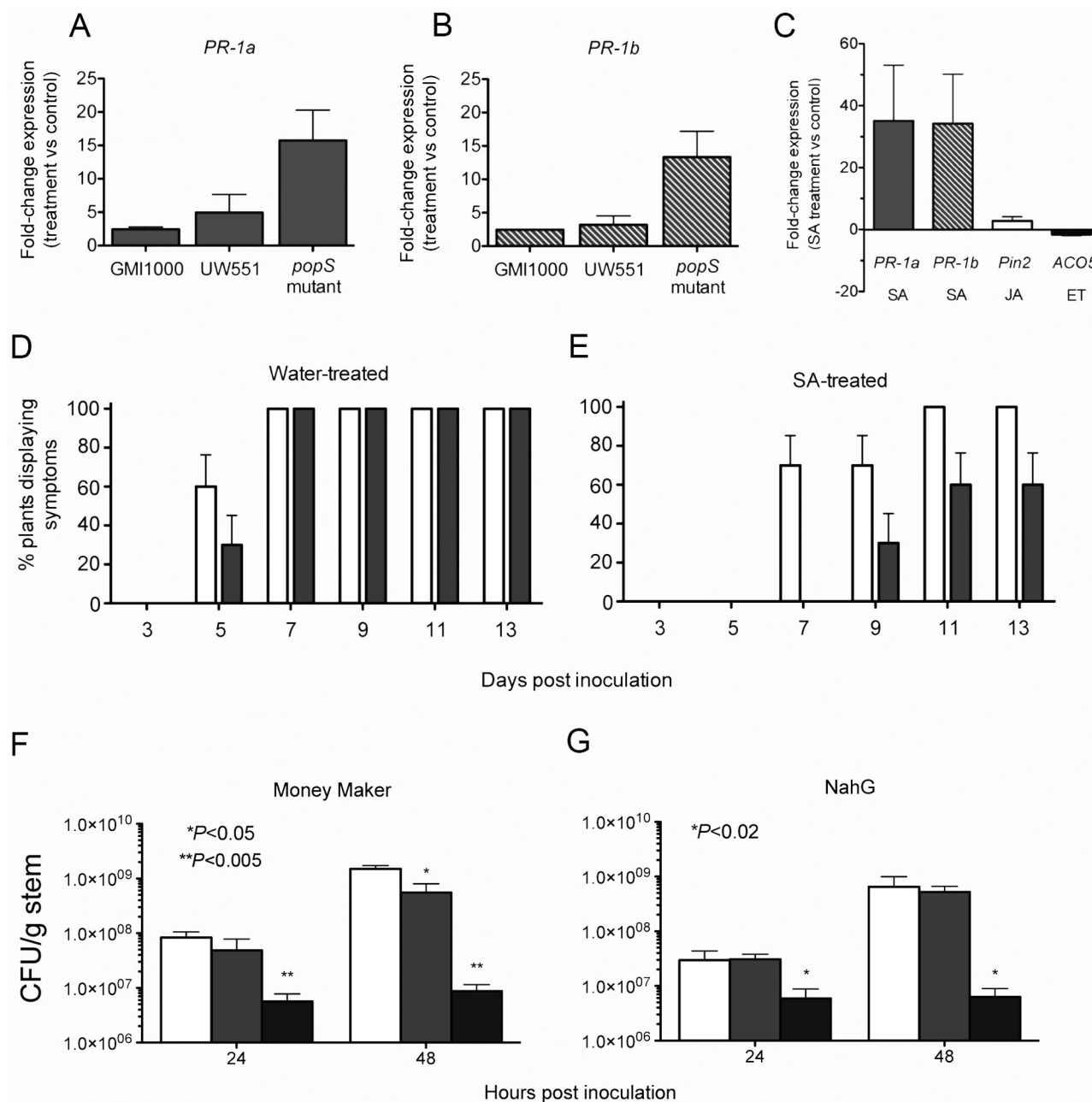


FIG 4 PopS is required to overcome SA-mediated defense induction. (A and B) Expression of tomato SA-induced *PR* defense genes increases in response to a *popS* mutant of *R. solanacearum*. Quantitative reverse transcriptase PCR was used to measure expression of plant defense genes in roots of the resistant tomato strain H7996 24 h after inoculation with *R. solanacearum* phylotype I strain GMI1000, phylotype II strain UW551 (WT), or the UW551 *popS* mutant. Expression of *PR-1a* (A) and *PR-1b* (B) was normalized to that of the tomato *GAPDH* gene, and the change in expression was determined using the $\Delta\Delta C_T$ method comparing pathogen-treated plants to water-inoculated control plants. Results reflect two replicates, each including 10 to 13 pooled roots per treatment; error bars indicate standard errors. (C) Expression of tomato defense genes *PR-1a*, *PR-1b*, *ACO5*, and *pin2* was measured by qRT-PCR 6 h after soil soak treatment of Money-maker tomato plants with 0.75 mM SA; RNA was extracted from midstem tissue, and expression levels are shown relative to those of water-treated controls. Results shown are the averages of two replicates, each including 5 pooled stem samples per treatment; bars indicate standard errors. (D and E) Treating plants with SA exacerbates the virulence delay of the *popS* mutant. Average symptom development of susceptible tomato plants (cv. Bonny Best) that were soil soak inoculated with approximately 1×10^8 CFU/g soil of *R. solanacearum* strain UW551 (WT) (open bars) or the UW551 *popS* mutant (filled bars). Six hours preinoculation, roots of the plants were drenched with either water (D) or 0.75 mM sodium salicylate (E) (10 plants per strain per treatment). A representative of two replicates is shown. By the end of the assay, all plants treated with water and WT UW551 or the *popS* mutant or with SA and WT UW551 were completely wilted, and 40% of plants treated with SA and the UW551 *popS* mutant were asymptomatic ($P = 0.0336$; Student's *t* test). Of these, 75% were colonized with $>10^{10}$ CFU/g and 25% contained no detectable bacteria. (F and G) Stems of susceptible cv. Money-maker tomato plants (F) or a SA-deficient NahG transgenic derivative of Money-maker (G) were inoculated through cut petioles with 40,000 cells of wild-type *R. solanacearum* strain UW551 (white), a *popS* effector mutant (gray), or a T3SS-deficient *hrcC* mutant (black). Columns represent the average bacterial population sizes (CFU/g stem) of 5 plants per treatment per time point, determined by dilution plating ground stem tissue 24 and 48 hpi; error bars indicate standard errors. Asterisks represent statistically significant differences between wild-type UW551 and the *popS* mutant or wild-type UW551 and the *hrcC* mutant (Mann-Whitney test).

Interestingly, pretreatment with SA significantly exacerbated the *popS* mutant virulence defect. SA-treated plants that were inoculated with the *popS* mutant never reached WT levels of disease ($P = 0.0336$; Student's *t* test) (Fig. 4E). In fact, 40% of SA-treated tomato plants inoculated with the UW551 *popS* mutant remained asymptomatic. Quantification of bacterial populations in these plants showed that three of the four asymptomatic plants harbored large *R. solanacearum* populations (average, 10^{10} CFU/g stem) but the remaining plant contained no detectable *R. solanacearum* cells. This observation was consistent across replicates (data not shown). In response to the *popS* mutant, SA-primed roots had decreased rates of initial stem infection and also delayed symptom development. These SA treatment experiments offer further evidence that *R. solanacearum* uses PopS to overcome SA-induced defenses.

SA-deficient NahG tomato plants restore the colonization defect of the *popS* mutant. If the function of PopS is to repress SA-mediated defenses, then reduced levels of SA *in planta* should allow a *popS* mutant to be more successful. We tested this hypothesis using transgenic Money-maker tomato plants expressing *nahG*, which encodes a bacterial salicylate hydroxylase that degrades salicylic acid and reduces SA-mediated defenses (40–42). We measured growth of WT UW551, the *popS* mutant, and the T3 secretion-deficient *hrcC* mutant in petiole-inoculated stems of wilt-susceptible cv. Money-maker and an isogenic SA-deficient NahG transgenic line. Both the *popS* and *hrcC* mutants were significantly delayed in colonization of nontransgenic Money-maker ($P < 0.05$ and $P = 0.004$, respectively; Mann-Whitney test) (Fig. 4F); after 48 h, the *popS* mutant grew to 5.6×10^8 CFU/g stem, compared to 1.5×10^9 CFU/g stem for the wild-type strain (Fig. 4F). These results demonstrated that Money-maker and the susceptible cultivar Bonny Best respond similarly to these *R. solanacearum* strains (Fig. 3C). The *hrcC* mutant grew equally poorly in both tomato lines, indicating that absence of SA alone is not enough to restore the stem growth defect of a completely T3SS-deficient strain (Fig. 4F and G). However, the UW551 *popS* mutant grew as well as its wild-type parent in the NahG tomato plant stems (Fig. 4G). Thus, an SA-deficient plant host could restore the *popS* mutant's colonization rate to wild-type levels, offering independent evidence that a direct or indirect function of PopS is to suppress SA-mediated plant defenses.

PopS does not elicit cell death in *Nicotiana benthamiana*. Some AvrE orthologs possess not only the ability to suppress SA-mediated defenses but also to cause cell death when they are expressed transiently in leaf tissue of *Nicotiana benthamiana* (14, 43). AvrE-like proteins contain conserved WXXXE motifs; at least two of these motifs are required to trigger cell death or for virulence (14, 43). A multiple alignment of PopS and other AvrE orthologs revealed that PopS contains all conserved tryptophans shown to be important for function in other AvrE family members (Fig. 5A) (14, 43). To determine if *R. solanacearum* PopS elicits plant cell death, we transiently expressed a C-terminally hemagglutinin (HA)-tagged PopS (PopS-HA) via *Agrobacterium tumefaciens* leaf infiltration of both tobacco and *N. benthamiana*. As a positive control, we infiltrated leaves to transiently express cell death-eliciting DspE from the necrotrophic soft rot bacterium *P. carotovorum* subsp. *carotovorum* (DspE_{Pcc}) (44). As expected, DspE_{Pcc} caused visible cell death at 24 hours postinfection (hpi) and complete tissue collapse at 48 hpi, but PopS did not elicit detectable cell death in *N. benthamiana* or *Nicotiana tabacum*

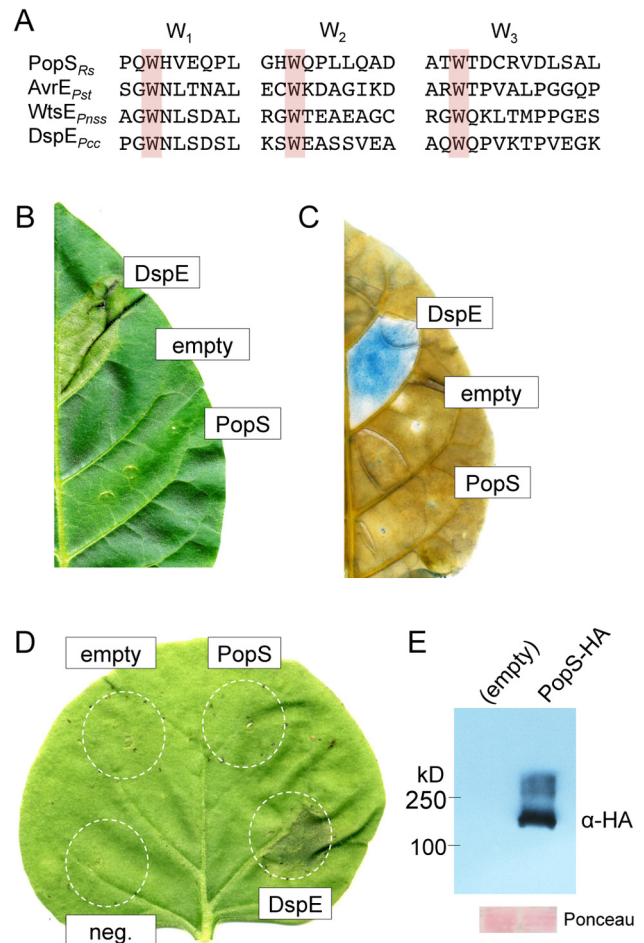


FIG 5 Transient expression of PopS in *N. benthamiana* leaves did not induce cell death. (A) Sequence alignment based on T-Coffee analysis of AvrE orthologs (PopS_{Rs}, AvrE_{Pst}, WtsE_{Pnss}, and DspE_{Pcc}). AvrE ortholog sequences from strains (NCBI sequence reference) analyzed include *R. solanacearum* UW551 (ZP_00944047.1), *P. syringae* pv. *tomato* DC3000 (NP_791204.1), *P. stewartii* subsp. *stewartii* (AAG01467.2), *P. carotovorum* subsp. *carotovorum* WPP14 (ZP_03833468.1). Conserved tryptophans known to be important for virulence or cell death activity (14, 43) are highlighted in red. (B to D) *Agrobacterium tumefaciens*-mediated transient expression in *N. tabacum* (B and C) and *N. benthamiana* (D). Leaves were infiltrated with *A. tumefaciens* pGWB14::popS (PopS-HA), *A. tumefaciens* pGWB2::dspE (DspE from *P. carotovorum* subsp. *carotovorum* [Pcc] WPP14) as a positive control, *A. tumefaciens* pGWB14 (empty vector control), or buffer as a negative control (neg.). Plant symptoms were imaged 48 h postinoculation. In panel C, trypan blue staining shows cell death caused by DspE. Each infiltration was repeated for at least three biological replicates. (E) Western blot analysis of PopS-HA (pGWB14::popS) or negative control (pGWB14; empty) from *N. benthamiana* leaf tissue.

(Fig. 5B, C, and D). An N- and C-terminally green fluorescent protein (GFP)-tagged version of PopS also did not elicit cell death (data not shown). Expression of PopS-HA in *N. benthamiana* was verified with Western blot analysis (Fig. 5E).

DISCUSSION

It is well established that T3-secreted effectors are essential for *R. solanacearum* virulence (16), but the biological roles of specific effectors remain underexplored. In this study, we characterized the function of PopS, an AvrE family effector that is present

throughout the *R. solanacearum* species complex. This effector family is widely conserved among plant-pathogenic bacteria, but its members make various contributions to pathogenesis (12–14, 45). In enteric plant pathogens such as *E. amylovora* and *P. carotovorum*, disrupting *dspE* renders the pathogen completely avirulent (44–46). In contrast, *avrE* mutants of *P. syringae* pv. *tomato* have no detectable colonization or virulence defects, although AvrE apparently works with other effectors, such as HopM1, to suppress host immunity and facilitate pathogenesis (12). PopS falls in the middle of this functional spectrum, because *popS* mutants are significantly delayed in virulence and plant colonization but can still cause bacterial wilt disease. The virulence and colonization defects of the *popS* mutant suggest that none of *R. solanacearum*'s more than 70 putative effectors is fully redundant with PopS activity (2, 22, 47). Nonetheless, a completely T3SS-deficient *hrcC* mutant was much less able to colonize plants than the *popS* mutant, confirming that additional T3-secreted effectors contribute to this process. As shown for *P. syringae* pv. *tomato*, multiple effector polymutants may identify those effectors that promote colonization and wilt in the absence of PopS (5, 13). Overall, the AvrE family's wide conservation and consistent role in virulence suggest that this effector has ancient origins in the evolutionary history of bacterial plant pathogens.

We determined that PopS contributes measurably to *R. solanacearum* virulence on several different hosts in the genus *Solanum*. Most strains of this broad-host-range pathogen have multiple effector families (e.g., GALA and AWR), whose homologs together potentiate virulence on solanaceous crop hosts such as tomato and eggplant (48–50). For example, individual GALA-family effectors are not required for full virulence on solanaceous hosts, but deleting three or more GALA effector genes delays wilt on tomato and eggplant (48, 49). PopS is a single-copy effector present in all members of the species complex, and our data indicate that it is needed for success on two agriculturally important *Solanum* hosts. Notably, PopS was dispensable for virulence on *S. dulcamara*, a common weed that can shelter and disseminate *R. solanacearum* (51). This difference suggests that PopS can have plant species-specific activity. As a result of selection pressures in natural ecosystems, wild hosts like *S. dulcamara* may have evolved to avoid PopS activity by modifying or eliminating the PopS target. Further studies are needed to define the specific mechanisms that permit *S. dulcamara* to resist PopS.

The *popS* mutant had the largest virulence defect on moderately wilt-resistant H7996 tomato, which upregulated its SA-induced *PR* defense genes to a greater degree in response to the *popS* mutant than in response to wild-type UW551. No such difference was observed in the response of roots of susceptible cv. Bonny Best (data not shown). We previously found that after infection by UW551, H7996 upregulates SA-mediated defense gene expression faster than Bonny Best (37). Consistent with this previous observation, we detected no differences in expression of *PR-1a* and *PR-1b* in Bonny Best roots inoculated with wild-type UW551 or the *popS* mutant (data not shown). We suspect that the larger virulence defect of the *popS* mutant on H7996 is directly correlated to the magnitude and timing of the defense signaling in H7996. This hypothesis was supported by our finding that susceptible tomato plants were more resistant to infection by the *popS* mutant when roots were pretreated with SA, which induces *PR* gene expression. It seems likely that PopS, like other AvrE-like effectors (12–14), also suppresses SA-induced immune responses,

such as callose deposition, that are triggered by recognition of MAMPs. The specific *R. solanacearum* MAMPs are undetermined, but purified *R. solanacearum* exopolysaccharide (EPS), a conserved and essential virulence factor, triggers increased *PR* gene expression in quantitatively resistant H7996 but not in susceptible Bonny Best (37). Further studies are needed to determine how *R. solanacearum*'s T3 effectors suppress MAMP- and EPS-triggered plant defenses.

The tomato pathogenesis-related protein *PR-1a* is triggered by SA (52). *PR-1b* has been described in the literature as ethylene responsive (53–55). Based on this, we previously used *PR-1b* as a marker of ethylene pathway activation (37). However, *PR-1b* has also been described as SA responsive (56, 57) and there is some evidence that it is upregulated under both conditions (52, 58, 59). We therefore directly tested the effect of SA exposure on expression of this gene in H7996 tomato. This experiment revealed that under our conditions, both *PR-1a* and *PR-1b* are upregulated around 35-fold in response to SA treatment (Fig. 4C).

The importance of PopS for tomato plant stem colonization and wilt is consistent with our previous observation that many *R. solanacearum* T3SS genes are highly expressed at midstage disease *in planta* (19). Further, SA-induced defenses are not expressed in tomato stems until *R. solanacearum* reaches 10⁸ CFU/g stem (37). Together these results affirm that T3 effectors are active not only at low pathogen cell densities early in colonization, as previously suggested (60, 61), but also at a later stage in the disease cycle. Between initial root infection and the end-stage collapse and death of the plant, *R. solanacearum* primarily inhabits the xylem elements, which are composed of nonliving tracheids; this raises the question of where T3SS effectors might act during midstage wilt disease. It has been suggested that bacteria in xylem elements inject effectors into the living xylem parenchyma cells that are adjacent to tracheids and accessible through the pits in xylem cell walls (19).

As expected (16), the T3SS-deficient UW551 *hrcC* mutant could not reach the 10⁸-CFU/g cell densities in stems required for bacterial wilt symptom development (62) and *hrcC* populations declined in tomato stems over time. This suggests that the T3SS is important not only for growth but also for persistence *in planta*. Both animal- and plant-pathogenic *Pseudomonas* spp. use the T3SS to persist in host tissue (63–66). Unlike the *popS* mutant, growth of the *hrcC* mutant was not restored in SA-deficient plants. We suspect that the constraints that limit success of the *hrcC* mutant include an inability to overcome basal immunity (1) or manipulate host physiology (4).

To the best of our knowledge, this is the first study to explore the defense-suppressing functions of an *R. solanacearum* effector in roots and stems, which are the important niches for *R. solanacearum* during natural pathogenesis. Using PopS as an example, we propose a model for effector functions during the bacterial wilt disease cycle where *R. solanacearum* uses T3 effectors for (i) root invasion and colonization, (ii) suppression of root defenses, (iii) stem colonization and growth, and (iv) induction of wilt symptoms. Further studies using adjustable promoters or inducible deletion mutations could reveal when these virulence traits are required during the disease process.

Three independent lines of evidence supported our conclusion that PopS suppresses SA-mediated defenses. First, a *popS* mutant strain elicited 3- to 4-fold-higher expression of SA-triggered *PR* genes in tomato than WT UW551. Second, pretreating tomato

plants with SA increased the magnitude of the *popS* mutant's virulence defect, as would be expected if that defect resulted from an inability to modulate SA-triggered plant defenses. Third, PopS was dispensable for colonization of SA-defective NahG transgenic tomato, as would be predicted if the *popS* mutant's colonization defect was caused by SA-mediated plant defenses. These results suggest that AvrE-family effectors generally function to suppress SA-mediated defenses, in beta- as well as gammaproteobacteria. Although PopS shares 23% amino acid sequence identity with its closest AvrE and DspE orthologs, protein sequence alignments revealed many scattered, moderately conserved regions, including the three conserved tryptophans important for the virulence activity in other gammaproteobacterial AvrE homologs (14, 43). Given the diversity of hosts that can be manipulated by AvrE-family proteins, it seems likely that this effector family interacts with a broadly conserved element of the plant defense system that indirectly or directly impacts SA-mediated responses.

Most surprisingly, we determined that PopS does not elicit cell death when transiently expressed in leaf tissue, unlike other AvrE homologs from hemibiotrophic and necrotrophic pathogens. Of the many AvrE-containing plant pathogens studied to date, *R. solanacearum* is the only one that causes a nonnecrotic wilt. As a biotroph, it multiplies to high cell densities in the xylem without causing necrosis. We speculate that as AvrE, WtsE, DspE_{Pcc}, and PopS diverged from a common ancestor, they adapted to the pathogenic lifestyles (necrotrophy, hemibiotrophy, and biotrophy) of each bacterium (Fig. 6). T3 effectors from hemibiotrophic *Pseudomonas* induce cell death in host tissue more often than effectors from biotrophic *R. solanacearum* (67), which suggests that in general, T3 effectors may function differently based on a pathogen's lifestyle. Necrotrophs such as *P. carotovorum* subsp. *carotovorum* kill host tissue upon plant contact and during multiplication. *P. carotovorum* subsp. *carotovorum* DspE_{Pcc} elicits cell death but does not suppress SA-mediated defenses (43, 44) (Fig. 6), which is consistent with the observation that SA is not a major signal associated with necrotroph infection. AvrE-possessing hemibiotrophs (e.g., *P. syringae* pv. *tomato* and *P. stewartii* subsp. *stewartii*) multiply in living host tissue but elicit necrosis during pathogenesis. Their AvrE homologs (AvrE and WtsE, respectively) not only elicit plant cell death when heterologously introduced into plant tissue but also indirectly or directly suppress SA-mediated defenses, which are known to defend plants against biotrophs and hemibiotrophs (Fig. 6) (12, 14; S. Y. He, personal communication). It remains to be determined if *P. syringae* HopR, the closest ortholog of PopS, causes cell death like AvrE, although it also suppresses SA-mediated defenses (13). *R. solanacearum* does not cause necrosis during infection and wilt, placing it on the biotrophic end of the spectrum. PopS does not elicit plant cell death but suppresses SA-mediated plant defenses, consistent with the biology of *R. solanacearum*. Functional studies of chimeric AvrE homologs and heterologous complementation across pathogens could reveal the specific domains that contribute to the distinct and common biological activities of this conserved effector.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. *Escherichia coli* was grown at 37°C in Luria-Bertani medium (68). *R. solanacearum* was cultivated at 28°C on rich Casamino Acids-peptone-glucose (CPG) medium (pH 7.0) (69). When required, media were supplemented with kanamycin (Km) (25 µg/ml),

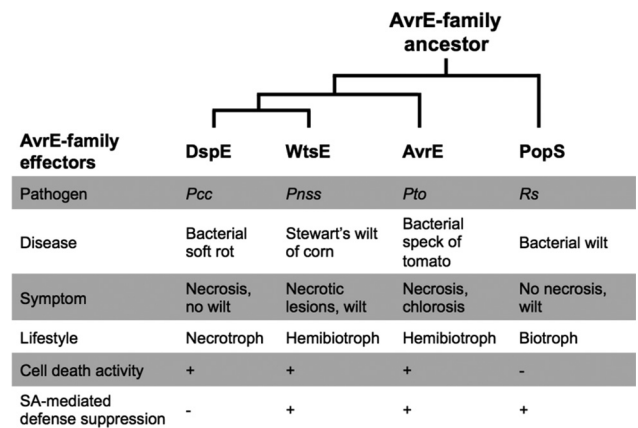


FIG 6 Functional phylogeny of the AvrE effector family. The phylogenetic tree is adapted from Fig. 1. DspE from the necrotroph *P. carotovorum* subsp. *carotovorum* (*Pcc*) causes cell death when transiently expressed in plant tissue but does not suppress SA-mediated defenses (43, 44). WtsE and AvrE from the hemibiotrophs *P. stewartii* subsp. *stewartii* (*Pnss*) and *P. syringae* pv. *tomato* (*Pst*), respectively, cause cell death when heterologously introduced into plant tissue and suppress SA-mediated defenses against virulence (12, 14). *R. solanacearum* (*Rs*) is a nonnecrotic wilt pathogen. We found that PopS does not elicit cell death when expressed in plant tissue but does suppress SA-mediated defenses during tomato wilt. This model suggests that these effectors have undergone adaptation to the pathogenic lifestyles of their respective microbes.

gentamicin (Gm) (15 µg/ml), tetracycline (Tc) (15 µg/ml), or rifampin (Rif) (25 µg/ml).

Recombinant DNA techniques and mutagenesis. Genomic and plasmid DNA was isolated by standard protocols (68). *E. coli* and *R. solanacearum* were transformed as previously described (68). PCR primer sequences are listed in Table S2 in the supplemental material. To disrupt the *R. solanacearum* UW551 locus *RRSL_03375* (*popS*), flanking regions from *popS* and a kanamycin resistance (*Km*^r) cassette were amplified from UW551 genomic DNA and pSTBlue-1, respectively, using Phusion high-fidelity DNA polymerase (Finnzymes, Vantaa, Finland). The *Km*^r cassette was inserted between the *popS*-flanking fragments by splicing by overlap extension (SOE)-PCR (70). Similar methods were employed to create a *hrcC* mutant (*hrcC::Gm*^r), except that a *Gm*^r cassette amplified from vector pUCGM was inserted in the regions flanking *hrcC*. The resulting SOE-PCR product was gel purified, phosphorylated with T4 polynucleotide kinase (Promega, Madison, WI), and ligated into the EcoRV site of cloning vector pSUP202 (71) to create pSUP202-*popS::Km*^r. Wild-type UW551 was transformed with pSUP202-*popS::Km*^r, and double recombinant mutants were selected for *Km*^r but screened for Tc^s to ensure proper allelic exchange. To complement the *R. solanacearum popS::Km*^r mutant, *popS* and its upstream native promoter were amplified from the UW551 genome and directly inserted into pENTR/D-TOPO (Life Technologies, Carlsbad, CA) following the manufacturer's instructions. UW551 *popS* was transferred to complementation vector pUC18-miniTn7T-Gm-GW via LR Gateway cloning as described by the manufacturer's protocol (Life Technologies, Carlsbad, CA). *R. solanacearum popS::Km*^r mutant competent cells were transformed with pUC18-miniTn7T-Gm-GW and Tn7 helper plasmid pTNS1 as previously described (72). Mutations were confirmed with PCR.

Phylogenetic analysis. Phylogenetic trees of PopS and AvrE/DspE/HopR orthologs were created with MEGA5 (25). The amino acid or gene sequences of PopS orthologs from NCBI or MAGE databases were aligned with CLUSTAL-W, from which a maximum-likelihood phylogenetic tree was created. The *R. solanacearum* strains used in this analysis are listed in Table S2 in the supplemental material. The percentage of replicate trees in which individual orthologs clustered together in the bootstrap test (200 replicates) was calculated and noted at each branch. MUM index (MUMi)

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Genotype and characteristics	Source or reference
<i>Escherichia coli</i> Top10	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>lacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>recA1</i> <i>araD139</i> Δ(<i>ara-leu</i>)7697 <i>galU</i> <i>galK</i> <i>rpsL</i> <i>endA1</i> <i>nupG</i> λ ⁻ Sm ^r	Life Technologies
<i>Agrobacterium tumefaciens</i> GV3101	Gm ^r Rif ^r	75
<i>Ralstonia solanacearum</i>		
UW551	Wild-type geranium isolate; phylotype II, sequevar 1	76
RSW19	UW551 <i>popS</i> ::Km ^r Km ^r	This study
RSW35	UW551 <i>popS</i> ::Km ^r pUC18-miniTn7T-Gm-GW:: <i>popS</i> Km ^r Gm ^r	This study
RSW36	UW551 <i>hrcC</i> ::Gm ^r , type III secretion deficient, Gm ^r	This study
Plasmids		
pSTBlue-1	Cloning vector, Ap ^r Km ^r	EMD Bioscience
pSUP202	Cloning vector, Ap ^r Km ^r Tc ^r Cm ^r	71
pUCGM	Ap ^r Gm ^r	77
pENTR/D-TOPO	Cloning vector, Km ^r ; Gateway (Life Technologies)	Life Technologies
pGWB2	Expression vector, 35S promoter, Km ^r	78
pGWB14	Expression vector, 35S promoter, C-terminal HA, Km ^r	78
pUC18-miniTn7T-Gm-GW	Complementation vector, Gm ^r ; Gateway (Life Technologies)	79
pTNS1	Helper plasmid for transposition; Ap ^r	79
ECW34	pSUP202:: <i>popS</i> ::Km ^r Ap ^r Km ^r Cm ^r	This study
ECW35	pSUP202:: <i>hrcC</i> ::Gm ^r Ap ^r Gm ^r Cm ^r	This study
pENTR/D-TOPO:: <i>popS</i>	~6.5-kb fragment containing UW551 <i>popS</i> with its native promoter cloned into pENTR/D-TOPO; Km ^r	This study
pUC18-miniTn7T-Gm-GW:: <i>popS</i>	~6.5-kb fragment containing UW551 <i>popS</i> with its native promoter cloned into pUC18-miniTn7T-Gm-GW; Gm ^r	This study
pGWB14:: <i>popS</i>	~5.2-kb gene encoding UW551 PopS cloned into pGWB14; Km ^r	This study
pGWB2:: <i>dspE</i>	~4.9-kb gene encoding <i>Pectobacterium carotovorum</i> subsp. <i>carotovorum</i> DspE	43

distances for whole-genome analysis were computed for each pair of sequenced genomes of *R. solanacearum* using the web server (<http://genome.jouy.inra.fr/mumi/index.cgi>). Briefly, the MUMi estimated the genomic distances by considering divergence of the *R. solanacearum* core genome as well as a gain/loss of DNA segments (34). The *R. solanacearum* species complex tree was created from a MUMi distance matrix using neighbor-joining cluster analysis.

Plant assays. To evaluate pathogen virulence, pots containing individual unwounded plants were soaked with a water suspension of UW551 or the *popS* mutant to create a final inoculum density of 1×10^8 CFU/g soil. Hosts included 17-day-old susceptible tomato plants (cv. Bonny Best), 14- to 16-day-old moderately resistant Hawaii 7996 tomato plants, 21-day-old potato plants (cv. Russet Norkotah) grown from minitubers, and 18- to 19-day-old bittersweet nightshade plants (*Solanum dulcamara*). Each treatment contained 14 plants and was repeated three times, except potato, which represents one biological replicate. Inoculated plants were incubated at 28°C with a 12-h light cycle. To evaluate the impact of salicylic acid on disease development, susceptible tomato (cv. Bonny Best) plants were pretreated with either 50 ml water or 50 ml 7.5 mM sodium salicylate in water, followed 6 h later by soil soak inoculation with either UW551 WT or the *popS* mutant. Disease was rated daily on a disease index scale from 0 to 4 (0, no wilt symptoms; 1, 1 to 25% of leaves wilted; 2, 26 to 50% of leaves wilted; 3, 51 to 75% leaves of wilted; and 4, 76% to 100% of leaves wilted). Each assay from two independent experiments included 10 to 14 plants per treatment. The effect of salicylic acid on defense gene expression in tomato was measured by extracting RNA as described below from Money-maker tomato stems 6 h after soil soak treatment with either 7.5 mM SA or sterile water as described above.

To measure bacterial colonization, plant stems were inoculated by applying a droplet of bacterial suspension to the cut petiole of the first true leaf. Host plants used for colonization were susceptible Bonny Best and resistant H7996 tomato. Bacterial colonization was quantified daily by grinding and dilution plating stem segments on appropriate antibiotic

plates. Four or five plants were sampled for bacterial colonization each day per treatment in two biological replicates. To evaluate the impact of plant salicylic acid levels on stem colonization, we measured bacterial colonization of wilt-susceptible tomato cultivar Money-maker and transgenic Money-maker expressing NahG (42). The experiment included five plants per treatment in two biological replicates.

Plant defense gene expression. Seeds of Bonny Best or H7996 tomato were surface sterilized with 50 ml 10% bleach for 10 min, followed by an ethanol wash in 50 ml 70% ethanol for 5 min. Ethanol and bleach washes were performed in 50-ml conical tubes, and seeds were incubated on a shaker at 200 rpm at room temperature. Seeds were then rinsed 5 to 7 times with sterile water to remove residual ethanol. For uniform germination, seeds were stored at 4°C overnight in the dark in water and then germinated on 1% water agar for 48 h at room temperature in the dark. Germinated seedlings were transferred to plates containing 1% agar and 0.5× Murashige and Skoog basal salts medium plus Gambour's vitamins (MS medium) (MP Biomedicals, Santa Ana, CA) and incubated for 2 days at 28°C with a 12-h light cycle. Root tips were inoculated with 2 μl containing 2×10^6 CFU/ml of either GM11000, WT UW551, or the *popS* mutant. Plant root tissue was harvested 2.54 cm from the inoculation point 24 hpi, immediately frozen in liquid nitrogen, and stored at −80°C. Results are averages of data from 7 to 12 plants per treatment.

Tomato RNA was extracted and purified from pooled tissue samples ground in liquid nitrogen using an RNeasy minikit (Qiagen, Valencia, CA) following the manufacturer's instructions, except that initial flow-through was applied to column twice. RNA was eluted in 30 μl RT-grade water. RNA purity and quality were evaluated on a NanoDrop (Thermo Scientific, Wilmington, DE) and an Agilent bioanalyzer picochip (Agilent Technologies, Santa Clara, CA), respectively. Samples with high quality (as defined by RNA integrity numbers greater than 8.0) and high purity (as defined by $A_{260/230}$ and $A_{260/280}$ of >1.9) were used for analysis.

Plant defense gene expression from tomato roots and stems was measured using quantitative reverse transcriptase PCR (qRT-PCR). One mi-

rogram of total RNA per sample was reverse transcribed using Superscript III reverse transcriptase first-strand synthesis Supermix (Life Technologies, Carlsbad, CA) with oligo(dT) and random hexamer primers, following the manufacturer's protocol. qRT-PCR was performed in duplicate with 1× PowerSYBR green master mix (Life Technologies, Carlsbad, CA), 400 nM forward and reverse primers, and 50 ng cDNA template for a final volume of 25 μ l. The reaction conditions were as follows: 10 min polymerase activation and 40 cycles of 95°C for 15 s and 57°C for 1 min. Relative gene expression was quantified for the tomato defense genes *PR-1a* and *PR-1b* using previously described primers (52) and normalized to that of a reference gene (*GAPDH*). Relative expression of treatment compared to control was defined using the $\Delta\Delta C_T$ method (73).

Transient expression of PopS in leaf tissue. The *popS* gene was amplified as described above and inserted into Gateway vector pENTR/D-TOPO following the manufacturer's instructions (Life Technologies, Carlsbad, CA). The *popS* gene was inserted into expression vector pGWB14 for C-terminal HA fusion protein expression using LR cloning (Life Technologies, Carlsbad, CA). The resulting plasmid, pGWB14::*popS*, was confirmed with sequencing and transformed into *Agrobacterium tumefaciens*, followed by selection with the appropriate antibiotics. Leaves from ~30-day-old *N. benthamiana* and *N. tabacum* plants were infiltrated with either buffer control, *A. tumefaciens* pGWB2::*dspE_{pecc}* (positive control) (43), *A. tumefaciens* pGWB14 (empty vector control), or *A. tumefaciens* pGWB14::*popS*. Leaf symptoms were observed and captured by scanning leaves at 48 h postinoculation. To verify the visible cell death elicited by *DspE*, plant leaves were stained with trypan blue as previously described (74). Each treatment was carried out in triplicate over three independent experiments.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00875-13/-/DCSupplemental>.

Table S1, DOCX file, 0.1 MB.

Table S2, DOCX file, 0.1 MB.

Figure S1, DOCX file, 0.1 MB.

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